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ARTICLE

Monoamine oxidases are novel sources of cardiovascular oxidative stress in experimental diabetes[1]

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Abstract: Diabetes mellitus (DM) is widely recognized as the most severe metabolic disease associated with increased cardiovascular morbidity and mortality. The generation of reactive oxygen species (ROS) is a major event causally linked to the development of cardiovascular complications throughout the evolution of DM. Recently, monoamine oxidases (MAOs) at the outer mitochondrial membrane, with 2 isoforms, MAO-A and MAO-B, have emerged as novel sources of constant hydrogen peroxide (H_2O_2) production in the cardiovascular system via the oxidative deamination of biogenic amines and neurotransmitters. Whether MAOs are mediators of endothelial dysfunction in DM is unknown, and so we studied this in a streptozotocin-induced rat model of diabetes. MAO expression (mRNA and protein) was increased in both arterial samples and hearts isolated from the diabetic animals. Also, H_2O_2 production (ferrous oxidation – xylenol orange assay) in aortic samples was significantly increased, together with an impairment of endothelium-dependent relaxation (organ-bath studies). MAO inhibitors (clorgyline and selegiline) attenuated ROS production by 50% and partially normalized the endothelium-dependent relaxation in diseased vessels. In conclusion, MAOs, in particular the MAO-B isoform, are induced in aortas and hearts in the streptozotocin-induced diabetic rat model and contribute, via the generation of H_2O_2, to the endothelial dysfunction associated with experimental diabetes.

Key words: monoamine oxidases, experimental diabetes mellitus, oxidative stress, endothelial dysfunction, clorgyline, selegiline.

Résumé : Il est largement accepté que le diabète sucré est la maladie métabolique la plus grave, associée à une morbidité et une mortalité cardiovasculaires. La production de dérivés réactifs de l'oxygène (DRO) est une manifestation majeure entraînant des complications cardiovasculaires tout au long de la progression du diabète sucré. Récemment, les monoamine oxydases (MAO) de la membrane mitochondriale externe sous deux isoformes (MAO-A et MAO-B) se sont démarquées comme de nouvelles sources de production constante de peroxyde d'hydrogène (H_2O_2) dans le système cardiovasculaire par l'intermédiaire de la désamination oxydative des amines biogènes et des neurotransmetteurs. On ne sait pas si les MAO sont des médiateurs de la dysfonction endothéliale dans le diabète sucré, et c'est ce que nous avons étudié avec ce modèle de diabète induit par la streptozotocine chez le rat. L'expression des MAO (ARNm et protéines) augmentait dans les échantillons artériels comme dans les cœurs isolés d'animaux diabétiques. De plus, la production de H_2O_2 (test de l'oxydation des ions ferreux révélée par le xylénol orange) dans des échantillons d'aorte était nettement accrue et associée à un déficit de la relaxation dépendante de l'endothélium (études en bain d'organe). L'administration des inhibiteurs des MAO (clorgyline et sélégiline) a permis de diminuer la production de DRO de 50 % et est parvenue en partie à rétablir la relaxation dépendante de l'endothélium à une valeur normale dans les vaisseaux atteints. En conclusion, dans le modèle de diabète induit par la streptozotocine chez le rat, les MAO, en particulier l'isoforme MAO-B, sont induites dans les aortes et les cœurs des animaux et contribuent par la production de H_2O_2 à la dysfonction endothéliale observée dans un modèle expérimental du diabète. [Traduit par la Rédaction]

Mots-clés : monoamine oxydases, diabète sucré expérimental, stress oxydatif, dysfonction endothéliale, clorgyline, sélégiline.

Introduction

Diabetes mellitus (DM), the most severe metabolic disease associated with increased cardiovascular morbidity and mortality, is widely recognized nowadays as a serious threat to global health, owing to its escalating prevalence worldwide (Guariguata et al. 2014).

The generation of reactive oxygen species (ROS) is a major event that is causally linked to the development of cardiovascular complications throughout the evolution of DM; however, the pathophysiological mechanisms underlying both diabetic cardiomyopathy and diabetes-related endothelial dysfunction are far from being fully elucidated (Joshi et al. 2014).

Monoamine oxidases (MAOs) are ubiquitous dehydrogenases located at the outer mitochondrial membrane that catalyze the oxidative deamination of neurotransmitters and dietary amines with the constant generation of hydrogen peroxide (H_2O_2), ammonia, and aldehydes as by-products of their catalytic cycle (Shih et al. 1999; Edmondson 2014). Two isoenzymes with different tissue distribution and sensitivity to pharmacological inhibition are described: the MAO-A isoform, which is irreversibly inhibited by

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clorgyline, and the MAO-B isoform, which is irreversibly blocked by low doses of selegiline (reviewed in Bortolato et al. 2008).

For more than half century, MAOs have been recognized as major contributors to the pathogenesis of human neurodegenerative and depressive disorders, and both irreversible and reversible pharmacological inhibitors have unequivocally proven their therapeutical properties in these settings (Al-Nuaimi et al. 2012; Song et al. 2013; Youdim et al. 2006).

However, during the past decade, MAOs have also emerged as important mitochondrial sources of oxidative stress in the cardiovascular system (for an excellent recent review of the topic see Kaludercic et al. 2014*b*).

In the heart, MAO-related oxidative stress contributes to the cell death triggered by myocardial ischemia–reperfusion injury (Bianchi et al. 2005), to mitochondrial dysfunction, and to maladaptive evolution of cardiac hypertrophy to heart failure (Kaludercic et al. 2010, 2014*a*).

With reference to the vascular system, in-vitro or in-vivo application of the irreversible MAO-B inhibitor selegiline elicited vasodilation via an increase in the amount of NO in cerebral arteries (Thomas et al. 1998). Also, MAO-A was found to be overexpressed in basilar arteries isolated from spontaneously hypertensive rats, together with increased H_2O_2 generation, an effect that was sensitive to the irreversible MAO-A inhibitor clorgyline (Poon et al. 2010).

Information regarding the contribution of MAOs to oxidative stress in the setting of diabetes is rather limited. Using a streptozotocin model of experimental diabetes, Manni et al. (2013) reported increased MAO-A activity in cardiomyocytes in response to the up-regulation of the renin–angiotensin system. Similarly, we found increased H_2O_2 generation in aortic rings harvested from Zucker diabetic fatty rats (ZDF), a genetic model of type-2 diabetes, that significantly diminished in the presence of clorgyline (Sturza et al. 2014).

The aim of this study was to investigate the role of MAOs as contributors to oxidative stress in the streptozotocin-induced rat model of diabetes.

Materials and methods

Six-week-old male Wistar rats were purchased from the Cantacuzino Institute (Bucharest, Romania) and were acclimated for 2 weeks prior to the study. At the age of 8 weeks, diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ; 50 mg/kg body mass). Age-matched control rats received an equal volume of vehicle (0.01 mol/L citrate buffer, pH 4.5). Two days after the injection, a blood sample was collected from the tail vein to measure the blood glucose. Rats with a blood glucose level over 200 mg/dL were considered diabetic. Animals were housed under standard conditions (constant temperature of 22.5 ± 2 °C, humidity 55% + 5%, with a 12 h (light) – 12 h (dark) cycle). Diabetes developed over a 2 month period, and glycemia and body mass were systematically monitored. Twenty-four hours prior to the experiment, solid food was withdrawn but there was no limitation in water supply.

All experimental procedures used in this study were conducted in accordance with EU Directive 2010/63/EU and the Romanian Law No. 43/May 2014, concerning the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Research Ethics of "Victor Babeş" University for Medicine and Pharmacy.

All reagents used were of the highest quality available and were purchased from Sigma–Aldrich, Invitrogen, Applichem, or Abcam.

ROS measurement

Hydrogen peroxide production was assessed in aortic rings harvested from animals with or without diabetes in the presence, compared with the absence, of MAO inhibitors (30 min preincubation with 10 μmol/L clorgyline or selegiline) by means of the ferrous iron – xylenol orange oxidation (FOX) assay, using a PeroxiDetect Kit (Sigma–Aldrich). The principle of the assay is that peroxides oxidize Fe^{2+} to Fe^{3+} ions at acidic pH. The Fe^{3+} ion will form a colored adduct with xylenol orange (XO; 3,3′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]-*o*-cresolsulfonephthalein; sodium salt), which can be observed at 560 nm.

Organ-bath studies

Organ-bath experiments were performed in rat aortic rings in the presence of diclofenac (10 μmol/L). The concentration of phenylephrine used for preconstriction was adjusted to obtain a preconstriction level of 80% of the contraction elicited by KCl (80 mmol/L). Endothelium-dependent relaxation to cumulative concentrations of acetylcholine (Ach) was recorded in the presence or absence of MAO inhibitors (10 μmol/L clorgyline or selegiline).

Real-time polymerase chain reaction (RT-PCR)

Quantitative RT-PCR was performed as previously described (Sturza et al. 2013*a*). Primers against MAO isoforms were designed using the sequence information (5′–3′) for rat from the NCBI database. MAO-A: forward, TCT CAG GAT TGG CTG CTG CCA AAC; reverse, CAG GTG GAA ATG CAC CAC GGA ATG. MAO-B: forward, TGG GCC AAG AGA TTC CCA GTG ATG; reverse, AGA GCG TGG CAA TCT GCT TTG TAG. The sequence numbers from the NCBI database for the preparation of MAO-A and MAO-B primers were NM_173740.3 for MAO-A and NM_172778.2 for MAO-B. The housekeeping gene and its primers were as follows: *EEF2α*, forward 5′-GACATCACCAAGGGTGTGCAG-3′; reverse 5′-GCGGTCAGCACA CTGGCATA-3′. Total RNA was isolated from aortic rings and cells with the Total RNA Mini SI Isolation Spin-Kit (Applichem) and used for reverse transcription (Superscript III RT; Invitrogen). The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 60 s), and elongation (72 °C, 60 s).

Immunohistochemistry

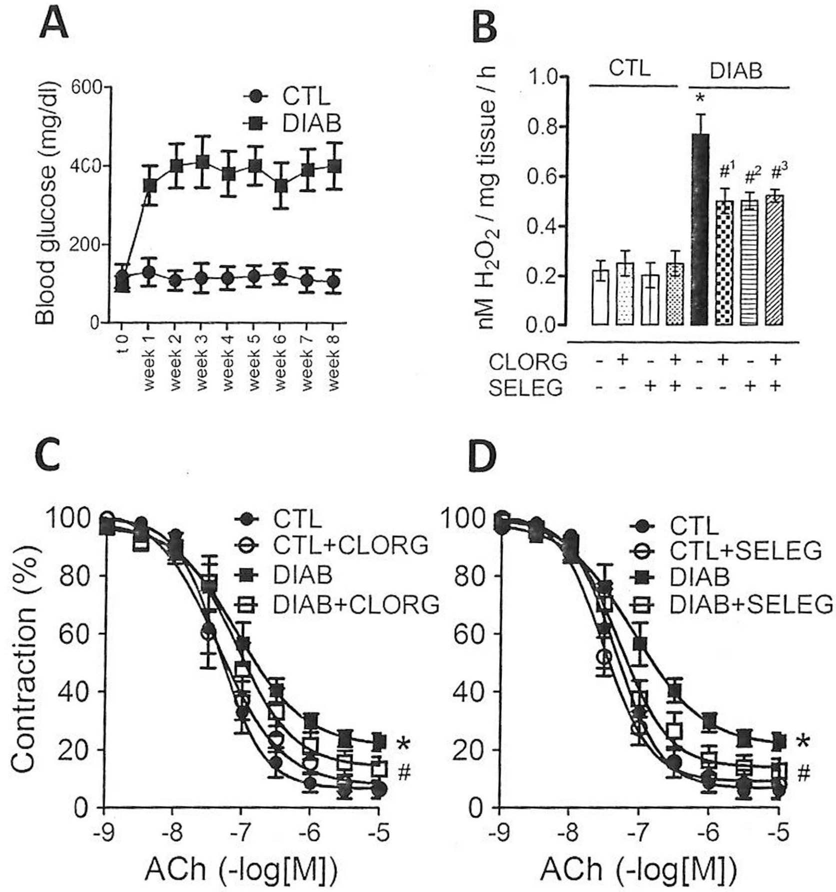
Tissue expression of the MAO isoenzymes was determined from frozen sections of rat hearts using MAO-A (ab126751; Abcam; 1:50) and MAO-B (ab125010; Abcam; 1:50) primary antibodies and Texas Red labeled secondary goat anti-rabbit antibody (SC2780,1:200; Santa Cruz), respectively. Nuclear staining was performed with DAPI (SC3598; Santa Cruz). The slides were examined on an Olympus Fluoview FV1000 confocal microscope (DAPI, excitation 405 nm and emission 461 nm; Texas Red, excitation 543 nm and emission 612 nm). Images were analyzed with Icy 1.6.1.1, a free open-source image analysis software developed by the Quantitative Image Analysis Unit at Institut Pasteur Paris (de Chaumont et al. 2012).

The expression of the isoenzymes in rat aortas was determined on formalin-fixed, paraffin-embedded tissue sections, using the same primary antibodies and Novolink Polymer Detection System (Novocastra, RE7150-K). These detection systems contain peroxidase block, protein block, post-primary block, Novolink polymer, DAB chromogen, Novolink DAB substrate buffer (polymer), and hematoxylin.

Statistics

Data are the mean ± SEM, and were analyzed using a one-way ANOVA or Student *t* test, where appropriate. Post-hoc comparisons among the groups was performed using the Tukey test. Data analysis of the dose–effect response curves was performed using the ANOVA *F* test (comparisons of bottom and top values, EC_{50}, and the Hill slope). Values of $p < 0.05$ were considered statistically significant.

g. 1. Monoamine oxidase (MAO)-mediated H_2O_2 production and endothelial dysfunction in diseased vs. normal vascular preparations.) Blood glucose levels in diabetic rats (DIAB) compared with the controls (CTL); $n = 6$ rats; *, $p < 0.05$. (B) Aortic H_2O_2 formation measured ing the ferrous oxidation – xylenol orange assay, $n = 6$ rats; *, $p < 0.05$ for DIAB vs. CTL; #[1–3], $p < 0.05$ for DIAB+MAO inhibitors vs. DIAB. (C ıd D) Acetylcholine-induced relaxation in phenylephrine-preconstricted rat aortic segments from the DIAB group and the CTL group in the ·esence or absence of clorgyline (C) or selegiline (D); $n = 6$ rats; *, $p < 0.05$ for DIAB vs. CTL. CLORG, clorgyline (10 μmol/L; MAO-A inhibitor); ;LEG, selegiline (10 μmol/L; MAO-B inhibitor); #, $p < 0.05$ with and without CLORG and SELEG.



Results

MAO is a mediator of endothelial dysfunction in the STZ-induced diabetic rat model

The role of MAOs as sources of ROS in the vascular system was studied in aortic rings harvested from diabetic rats and compared with the control animals. Experiments were performed 2 months after diabetes was induced, and the development of glycemia in the diabetic rats was compared with the control animals (Fig. 1A). As demonstrated with the FOX assay, the amount of H_2O_2 production was significantly higher in vascular segments isolated from the diabetic as compared with the non-diabetic group. Incubation for 30 min with the irreversible MAO inhibitors (10 μmol/L clorgyline or selegiline) partially restored the H_2O_2 levels (Fig. 1B). In separate experiments, the endothelium-dependent relaxation (EDR) of vascular segments from the diseased vs. the control animals was assessed. EDR was significantly attenuated in vascular rings from diabetic rats, and this effect was inhibited in the presence of either the MAO-A inhibitor clorgyline (Fig. 1C) or the MAO-B inhibitor selegiline (Fig. 1D). Of note, the drugs had no effect on vascular reactivity in the control segments.

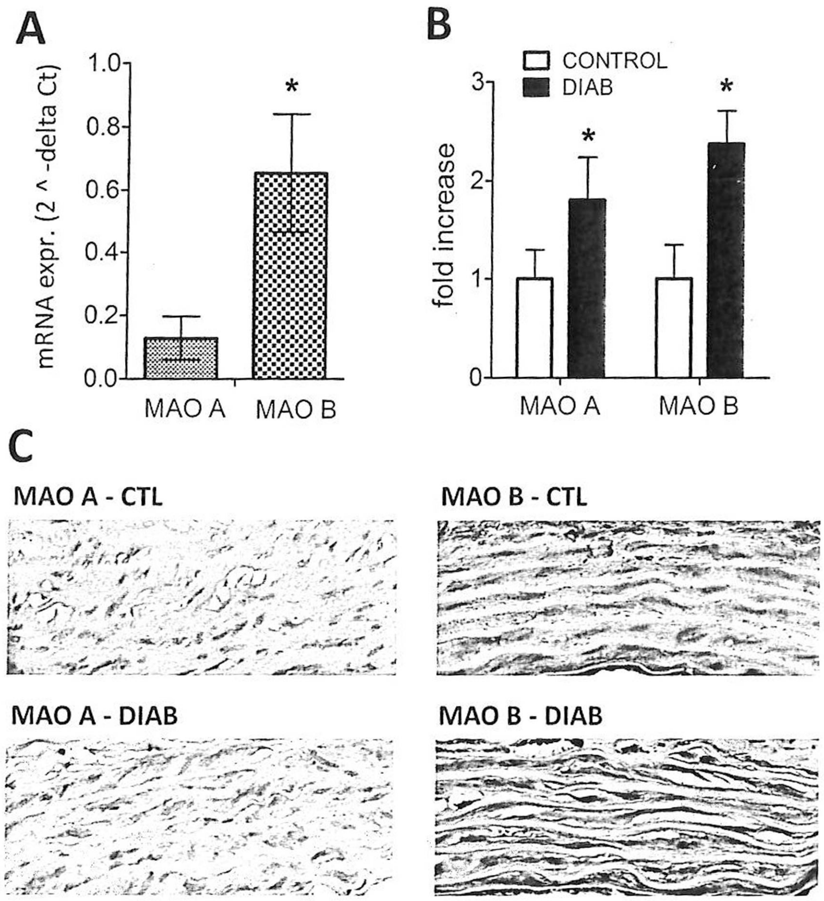
Vascular expression of MAO-A and -B isoforms is increased in the STZ-induced diabetic rat model

Since, in organ-bath experiments, incubation with either MAO inhibitor had no effect on vascular reactivity of the aortic rings harvested from the control group, we hypothesized that both isoforms are induced only in diseased animals. To address this hypothesis, we determined the expression of MAO-A and MAO-B by RT-PCR and immunohistology in aortas isolated from rats with or without diabetes. In non-diabetic animals, the expression of MAO-B isoform, as judged from the difference in C_T values as well as from the intensity of the DAB staining, was more abundant than the MAO-A isoform (Fig. 2A). mRNA expression of both MAO isoforms showed an almost 2-fold increase (Fig. 2B) in aortic segments isolated from STZ-induced diabetic rats compared with the controls. To determine whether mRNA induction also translates into changes in protein expression in aortic rings, immunohistology was also performed. As shown by the DAB staining in Fig. 2C, the MAO-B isoform was significantly more expressed in vascular rings harvested from diabetic animals as compared with the control group.

Myocardial expression of MAO-B (but not of MAO-A) isoform is increased in the STZ-induced diabetic rat model

To investigate whether the up-regulation of MAOs also occurs in the heart as well as the vessels, we further performed immunofluorescence studies in myocardial samples isolated from diabetic and non-diabetic rats. Interestingly, no difference in the expression of MAO-A isoform could be detected between the diseased and control animals (Fig. 3). However, the opposite results were found for the MAO-B isoform expression, where the intensity of the fluorescent signal was significantly higher in heart samples

Fig. 2. Monoamine oxidase (MAO) expression in vascular preparations from STZ-induced diabetic (DIAB) and non-diabetic (CTL) rats. Aortic segments from rats with or without diabetes. (A) Real-time (RT)-PCR (mRNA expression, $2^{-\Delta C_T}$) for MAO-A and MAO-B relative to the housekeeping gene *EEF2α* in aortic segments from control animals; $n = 4$ rats; *, $p < 0.05$. (B) RT-PCR (fold increase) for MAO-A and MAO-B relative to *EEF2α* in aortic segments from DIAB vs. CTL; $n = 4$ rats; *, $p < 0.05$. (C) Immunohistology for MAO-A and MAO-B in diabetic (DIAB) and control (CTL) aortic segments.



harvested from the diabetic rats by comparison with the controls (Fig. 4).

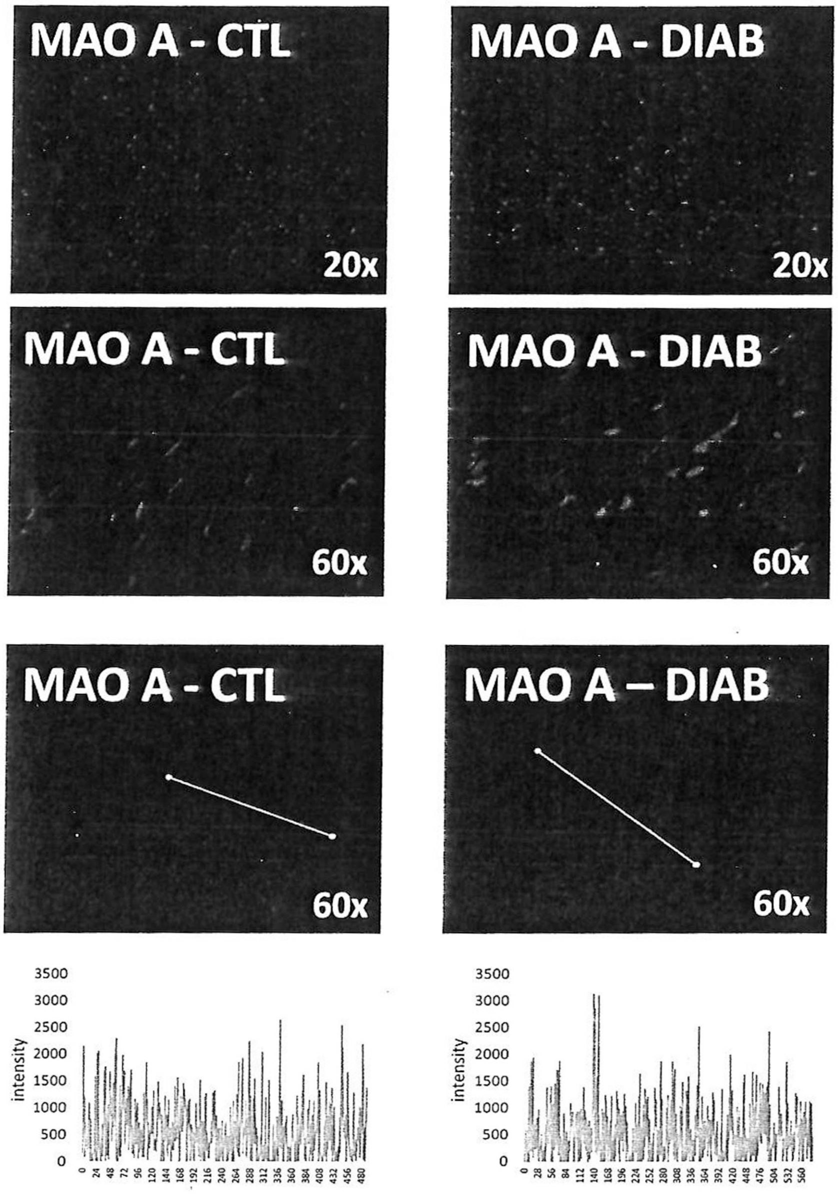
Discussion

The major finding of our study is that MAOs contribute to the development of oxidative-stress-mediated endothelial dysfunction in experimental diabetes.

From our organ-bath experiments, we report a significant reduction in the endothelium-dependent relaxation of vascular segments in diabetic animals compared with the controls. Incubation with the irreversible MAO inhibitors (clorgyline for MAO-A and selegiline for MAO-B) partially restored the endothelium-dependent relaxation, suggesting a role of both isoforms as mediators of endothelial dysfunction in this experimental model. This observation is in agreement with our previous findings in 2 murine models of acute (a single injection of lipopolysacharride, i.p.) and chronic (2 weeks administration of angiotensin II, via minipumps) oxidative stress. Accordingly, we reported that high levels of either lipopolysaccharide or angiotensin II led to overexpression of both MAO isoforms, with a subsequent increase in ROS production and impairment of vascular relaxation (Sturza et al. 2013*a*). In another study, we have also demonstrated that ex-vivo pretreatment with the same MAO inhibitors attenuated the endothelial dysfunction in aortic rings harvested from spontaneously hypertensive rats (Sturza et al. 2013*b*). It has been previously reported in the literature that treatment with angiotensin II converting enzyme inhibitors reduced MAO activity, suggesting that activation of the renin–angiotensin–aldosterone system may be responsible for the enzymatic induction (Raasch et al. 2002). Furthermore, in-vitro or in-vivo application of selegiline, the irreversible MAO-B inhibitor, elicited vasodilatation via an increase in the amount of nitric oxide in brain tissue and cerebral blood vessels (Shiva 2010).

We have also shown in this study that diabetes elicited a significant increase in the amount of H_2O_2 generated in vascular preparations via this mitochondrial enzyme (MAO) as determined by the FOX assay. In fact, type I experimental diabetes elicited a 4-fold increase in the H_2O_2 production in aortic rings, whereas incubation (30 min) with MAO inhibitors reduced the amount of ROS by 50%. These data are in agreement with those reported in an experimental model of type-2 diabetes, the Zucker diabetic fatty rat, where a significant (albeit less important as compared with the type-1 model of diabetes) H_2O_2 increase was found in the vascular segments. Similar to what we have shown here, ex-vivo incubation of aortic rings from Zucker diabetic fatty rats with MAO inhibitors diminished the oxidative stress by more than 50% (Sturza et al. 2014). Clearly, in both models of diabetes, the contribution of other mitochondrial and cellular sources of ROS to the oxidative stress cannot be ruled out. In the cardiovascular system, NADPH oxidases and xanthine oxidase are prominent sources of oxidative stress and were reported to be overexpressed in diabetes, with a subsequent increase in H_2O_2 production (Brandes and Schroder 2008; Xia et al. 2014).

Fig. 3. Monoamine oxidase (MAO)-A expression in heart samples from STZ-induced diabetic (DIAB) and non-diabetic (CTL) rats. Immunofluorescence: red, anti-MAO-A antibody; blue, DAPI.



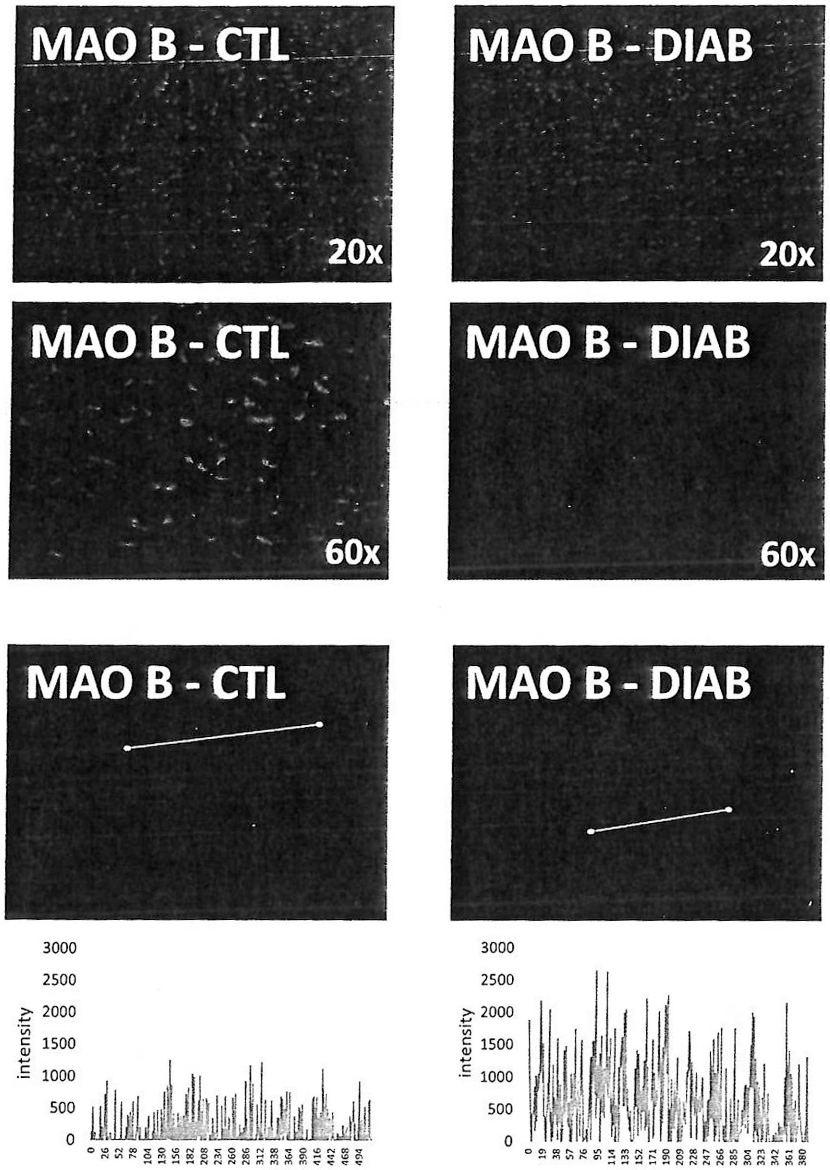
The alleviation of oxidative stress via MAO inhibition, however, raises 2 important questions: (*i*) do MAO inhibitors act as H_2O_2 scavengers, and (*ii*) is there is a cross-talk between MAO inhibitors and the other cellular ROS sources? In our previous study performed in mice, we have unequivocally proven that irreversible MAO inhibitors (clorgyline, selegiline) neither acted as H_2O_2 scavengers nor potentiated xanthine oxidase and NADPH oxidase (Nox1, Nox2, and Nox4)-driven H_2O_2 formation (Sturza et al. 2013*a*). In this model, we showed that the induction of vascular MAO is mediated by NF-κB and phosphatidylinositide 3-kinase, and proposed a pathophysiological sequence that could be responsible for the MAO-related endothelial dysfunction (Duicu et al. 2013).

Another important finding of this study is that in the control animals, MAO inhibition did not interfere with H_2O_2 production and vascular reactivity, suggesting that in basal conditions, MAO activity is reduced and the amount of ROS produced is low, and thus unable to impair endothelial function.

Last but not least, we showed here, for the first time, that MAO-B is the dominant isoform that is upregulated in both vascular preparations and heart samples harvested from diabetic animals. Both rat and human MAO-B have been reported to increase in the brain with advancing age (reviewed in Edmondson 2014); whether the same holds true for the vascular system remains to be demonstrated.

The increased generation of ROS in the cardiovascular system in the setting of diabetes has been systematically reported. Classically, the major pathways responsible for overproduction of ROS are represented by the following: the polyol pathway (Toth et al. 2007); the increased formation of advanced glycation end-products and their receptors (Singh et al. 2014); the activation of protein kinase C (PKC) (Kizub et al. 2014); and the overactivity of the hexosamine pathway (Giacco and Brownlee 2010). The cellular sources of ROS associated with these biochemical pathways are represented by NADPH oxidases, the respiratory chain, and eNOS uncoupling. Here we have demonstrated that MAOs with 2 isoforms,

Fig. 4. Monoamine oxidase (MAO)-B expression in heart samples from STZ-induced diabetic (DIAB) and non-diabetic (CTL) rats. Immunefluorescence: red, anti-MAO-B antibody; blue, DAPI.



A and B, are also important contributors to oxidative stress in experimental diabetes. However, in this respect we acknowledge at least 2 limitations of our study: first, we did not assess the effects of increased MAO on mitochondrial function; and second, the potentially deleterious effects of the other 2 by-products of MAO's catalytic cycle (ammonia and aldehydes) were not addressed. The former issue may become important in the setting of diabetes and in-line with the statement claiming that H_2O_2 generated via the MAO-B isoform could be more destructive than that produced by MAO-A (Edmondson 2014).

As previously mentioned, MAOs have been extensively studied for their involvement in the pathogenesis of neurodegenerative and psychiatric diseases (Yamada and Yasuhara 2004), and an important therapeutic armamentarium of MAO inhibitors are available for the treatment of several pathologies, including Parkinson's disease, affective disorders, stroke, and senescence (Carradori and Petzer 2015). On the other and, diabetes has been recently associated with depression (Gupta et al. 2014), Parkinson's disease, and Alzheimer's disease (Holscher 2014) as major complications of impaired neurotransmission. Bearing in mind that reversible MAO-B inhibitors represent an important class of drugs largely indicated for their beneficial effects (including their neuroprotective properties) in psychiatric and neurological diseases (Al-Nuaimi et al. 2012; Song et al. 2013), the observation that diabetes mainly increases the expression of the MAO-B isoform appears to be relevant as potential therapeutic target in this complex metabolic disease.

Interestingly, it has been recently reported that pioglitazone, a widely employed anti-diabetic drug, acts as a specific and reversible inhibitor of human MAO-B (Binda et al. 2011). Whether diabetic patients treated with pioglitazone are less prone to develop endothelial dysfunction remains to be further clarified.

Nevertheless, the hypothesis that the decrease in MAO-mediated tissue injury could be protective in human vascular and cardiac samples harvested from diabetic patients clearly warrants further investigation (Duicu et al. 2014).

Conclusions

In conclusion, while both isoforms of MAO are expressed in the rat cardiovascular system, it is the MAO-B isoform that is mainly induced in the experimental model of STZ-induced diabetes. MAO-induced endothelial dysfunction was partly mediated by H_2O_2, and ex-vivo inhibition of MAOs reasonably restored the normal function in diabetic vascular preparations. Monoamine oxidases are novel mediators of endothelial dysfunction in the experimental rat model of type-I diabetes.

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